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GRANT NUMBER DAMD17-96-1-6029

TITLE: Immunobiological Aspects of erbB Receptors in Breast Cancer

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REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

19981030 056

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Annual (15 Jul 97 - 14 Jul 98)

4. TITLE AND SUBTITLE Immunobiological Aspects of erbB Receptors in Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6029

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pennsylvania
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Our work is concerned with the erbB-2/neu family of receptors. The purpose of these studies was to provide information on the role of the endodomain in signalling and endocytosis of the receptor complex. In addition, we wished to identify, molecularly, ligands that would specifically bind to and activate the neu receptor. We have significantly increased our understanding of how the p185neu endodomain associates with and contributes to EGFR-p185 heteromeric receptor signalling and endocytosis. We used a variety of molecular mutants and chimeras (EGFR ectodomain and neu transmembrane and endodomain) to characterize specific residues and subdomains of the endodomain responsible for discrete activities. In addition, we have used an oligonucleotide probe derived from the transmembrane sequence of erbB-3 and 4 ligands to screen an ATL-2 cDNA library and have identified novel ligand sequences that potentially may represent an authentic p185neu ligand. These sequences will be used to identify a full length cDNA sequence that will be tested for biological activity in vitro and in vivo. The significance of these findings is that they provide new insights into the structure and behavior of this receptor family and its ligands in normal cell growth and breast cancer development.

19981030056

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer ErbB receptors	23
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The ErbB family includes four members of homologous receptor tyrosine kinases (RTKs): the epidermal growth factor receptor (EGFR or ErbB-1) (1), ErbB-2/p185$^{c\text{-}neu}$ (2, 3), ErbB-3 (4), and ErbB-4 (5). ErbB family proteins are widely expressed in epithelial, mesenchymal, and neuronal tissues and play important roles in normal growth and development (6-9). Aberrant expression of these erbB proteins is frequently observed in human malignancies [reviewed in (10)].

The transmembrane mutation in rat p185^{neu} (11) serves as a paradigm for receptor dimerization as a means of constitutive kinase activation contributing to oncogenic transformation (11-13). Additional support for this mechanism has come from the identification of a naturally occurring activated EGFR oncoprotein (ΔEGFR or EGFRvIII) in human tumors which forms constitutive dimers and confers increased tumorigenicity (14, 15). p185$^{erbB\text{-}2}$ tyrosine kinase activity is induced as a result of receptor oligomerization, and gene amplification and overexpression of erbB-2 have been observed in a high frequency of human adenocarcinomas including those of the breast and ovary. These features correlate with poor clinical prognosis (16, 17). Experimental support for this model is provided by in vitro transformation assays using cell lines overexpressing either protooncogenic rat p185$^{c\text{-}neu}$ or human erbB-2 at levels of 10^6/cell (18, 19). Biochemical and biophysical analysis of baculovirus-expressed p185$^{c\text{-}neu}$ proteins further supports the notion of receptor oligomerization as a mechanism of kinase activation of normal holo-receptors (20, 21).

Heterodimeric interactions govern many signalling properties within the erbB receptor family. Co-expression of the EGFR and p185$^{c\text{-}neu}$ at modestly elevated levels (10^5/cell) (but not either receptor independently) results in transformation (22) due to an increase of the ligand binding affinity and catalytic kinase activity (23, 24). Heterodimerization of the EGFR and erbB-2 has been observed in human breast tumor lines (25). Moreover, ligand treatment promotes the assembly of an activated p185$^{c\text{-}neu}$/EGFR kinase complex (24), resulting in novel distinct cellular signalling events (26). Therefore, the receptor tyrosine kinase ensemble can be activated not only by homodimer formation, but also by heterodimeric association. In this regard, endodomain interactions between p185$^{c\text{-}neu}$ and the EGFR appear to influence functional signalling outcomes (27).

In response to EGF or NDF/HRG (Neu Differentiating Factor/Heregulin, a ligand for erbB-3 and erbB-4) family ligands (28, 29), the EGFR and erbB-2 both form heterodimers with erbB-3 and erbB-4 (30-34). Heterodimers between erbB-2 and erbB-3 are associated with activated signalling and the transformed phenotype in primary human cancer cells (35). Existence of an erbB-3-erbB-4 heterodimer has not been demonstrated to date. More recent data support the notion that erbB-2 is the preferred heterodimerization partner of *all* erbB receptors and a mediator for divergent cellular signalling in many distinct cell types (34, 36).

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Neuregulin-1 (NRG-1) was identified in several tissue contexts [as NDF (69), as GGF (70), and as ARIA(71)] as a gene encoding a growth factor that binds directly to the erbB-3 and erbB-4 receptor tyrosine kinases. Many forms of the NRG-1 gene product exist primarily as a result of alternative splicing. Alpha and beta forms of neuregulin-1 exist that differ in one region in the EGF-like domain that distinguishes these neuregulin forms based on their ability to affect neural (beta forms) verses mesenchymal (alpha forms) cell types. Neuregulin-2 (NRG-2) was identified using reduced stringency conditions and NRG-1 probes (64) and by using NRG-1 primers and RTPCR (65, 72). Like NRG-1, NRG-2 was also found to contain the same 4 variable regions, suggesting the existence of multiple forms of NRG-2, and was found in a partially overlapping yet distinct set of tissues in the developing and adult animal as compared with NRG-1. All of the protein forms of NRG-2 so far studied were also found to bind directly to the erbB-3 and erbB-4 proteins. To date, none of the forms of either NRG-1 or NRG-2 that have been found directly bind and activate the erbB-2 (neu) gene product.

In previous work we characterized a neu/erbB-2 protein-specific activating factor designated NAF found to be a product of a unique HTLV-1 transformed T cell leukemia cell line, ATL-2, originally produced and provided to us by the Ajinomoto Corporation. NAF has the ability to bind to the extracellular domain of p185c-neu and promote kinase activation and dimerization with other p185 holoreceptors (66).

Although numerous splice variants have been detected, the several forms of the NRG protein that have been studied biochemically were found to bind directly only to the erbB-3 and erbB-4 RTKs (64,65,72). Biochemically, the various NRG forms can have similar or contrasting physiologic affects when compared with one another. There are still distinct isoforms of NRG-1 being identified (67). It is possible that other isoforms of the NRG-2 (or NRG-1) protein exist that may bind to other erbB RTK family members. It is also possible that other neuregulin-related genes exist.

The objectives of these studies involved two major aims. The first aim was to gain a better understanding of the formation of homomeric or heteromeric ensembles of the $p185^{neu}$ or $p185^{c\text{-}neu}$ polypeptides and EGF receptors. The focus of this part of the effort was on the role of the endodomains of these molecules. The second aim was to clone the authentic ligand of the p185 molecules. We have made significant progress in both areas.

BODY

The structural basis for erbB receptor heterodimerization has not been completely defined and crystallographic information on dimerized erbB receptor kinases is currently unavailable. Previous work has revealed that ectodomain interactions are sufficient to stabilize dimer formation between $p185^{neu}$ and the EGFR in fibroblasts and transformed cells (37, 38).

We have constructed various p185^{neu} deletion mutants in order to compare signalling events resulting from associations between holo-EGF receptors and either p185^{neu} ectodomain- or endodomain-derived mutant receptors. Enhanced, diminished or unchanged signalling outcomes resulting from heterodimeric EGFR and mutant p185^{neu} associations have revealed the functional importance of p185^{neu} subdomains in heteroreceptor signalling contributing to receptor ensemble internalization, cell growth and transformation.

Experimental Methods:

Antibodies

Monoclonal antibody (mAb) 7.16.4, anti-Bacneu, or NCT reactive with the ectodomain, intracellular domain, or carboxyl terminus of p185neu, respectively, have been described previously (20, 39, 40). mAb 425 reactive with the ectodomain of EGFR was obtained from Dr. John Mendelsohn (Memorial Sloan-Kettering Cancer Center, New York, N.Y.). A polyclonal rabbit antiserum specifically against the C-terminus of EGFR (termed CT) was provided by Dr. Stuart Decker (41). The anti-phosphotyrosine monoclonal antibody, PY20, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

DNA Constructs

All the new deletion mutants were derived from the rat oncogenic p185^{neu} cDNA containing a single point mutation (V664G) in the transmembrane region. The TAPstop mutant, containing a 122-aa truncation of the C-terminus was made as described (42). The T691stop was made by site-directed mutagenesis and substitution of a stop codon for Thr691, resulting in a large cytoplasmic deletion (37, 38). The ectodomain-deleted mutant TΔ5 neu protein was described previously (27). These cDNAs encoding for mutant p185^{neu} forms were all cloned into the pSV2neo^{r}/DHFR vector as described (37) for eukaryotic expression. The pSRaEGFR/hyg^{r} vector (43) was used for full-length EGFR expression. We have also prepared a variety of chimeric receptors using the ectodomain of the EGFR and the transmembrane and various endodomain mutants of neu. We have prepared Er/Tneu, Er/T691 stop, and Er/ TAPstop as described (37,43).

Transfection and maintenance of cell lines

Ten micrograms of various p185^{neu} constructs were transfected into NR6 cells [a mouse fibroblast cell line devoid of endogenous EGF receptors (44)], or NE91 cells [which express the human EGFR (37)] by calcium phosphate precipitation. After 2-3 weeks selection with geneticin (0.9 mg/ml), the established stable clones were screened and characterized. Gene amplification by methotrexate was used to increase the p185^{neu} receptor level. Expression of p185^{neu} and its derivatives in resultant subclones were examined by flow cytometric analysis following anti-p185^{neu} mAb 7.16.4 staining. Surface expression of p185^{neu} proteins was then estimated by comparing the mean channel fluorescent intensity with that from

B104-1-1 cells, as the level of $p185^{neu}$ on B104-1-1 cells was previously determined by the ^{125}I-labeled anti-neu mAb binding assay (22). EGFR numbers in NE91 cells and mutant $p185^{neu}$ co-transfected cells were determined by Scatchard assays as described (43). These transfected clones were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS, Hyclone) at 37°C in a 5 % CO_2 atmosphere.

Cross-linking, immunoprecipitation, and immunoblotting procedures

Subconfluent cells in 10-cm dishes were washed and starved in cysteine-free DMEM for 1 hour, and grown in low cysteine-containing 5% FBS-DMEM containing 55 μCi/ml of [^{35}S]-cysteine (Amersham) for 16 hours for metabolic labeling. Alternatively, the unlabeled cells were cultured overnight in 10-cm tissue culture dishes. After treatment with or without EGF, cells were washed twice with cold phosphate-buffered saline (PBS) and treated with PBS containing a 2 mM concentration of the membrane impermeable cross-linker bis(sulfosuccinimidyl) suberate, BS^3 (Pierce), for 30 min. After quenching the cross-linking reaction with a buffer containing 10 mM Tris-HCl (pH 7.6), 0.9% NaCl and 0.1 M Glycine, cells were washed twice with cold PBS and solubilized with PI/RIPA buffer as described (24). The immunocomplexes were washed and solubilized, then separated by gradient SDS-PAGE gels (4-7.5%). Proteins from metabolically-labeled cells were analyzed by autoradiography. Proteins from unlableled cells were transferred onto nitrocellulose, then immunoblotted with either anti-phosphotyrosine mAb PY20, anti-EGFR CT or anti-p185 antiserum as indicated in the figures. The protein signals were identified by the binding of [^{125}I]-protein-A (NEN).

In vitro transformation assays

Equal numbers of cells were plated in tissue culture dishes in triplicate and cultured in DMEM containing 2% FBS. The media was changed every 3-4 days. After 21-days in culture, cells were fixed with 10% formalin and stained with hematoxylin to observe morphologically transformed foci. Anchorage-independent growth ability was determined by assessing the colony forming efficiency of cells suspended in soft agar (43, 45). Cells (1000 per dish) were suspended in 7% FBS-DMEM containing 0.18% agarose, and plated on 0.25% of basal agar in each dish. Cells were fed with DMEM supplemented with 7% FBS-DMEM, 20 mM Hepes (pH 7.5). Colonies (>0.3 mm) were visualized at day 21 for all cell lines after being stained with *p*-iodonitrotetrazolium violet (1 mg/ml). Each cell line was examined in triplicate samples for separate experiments.

Receptor down-regulation studies

Cells (1×10^5) were incubated overnight in 6-well dishes with DMEM containing 5% FBS. Cells were then treated with EGF (50 ng/ml) for 0-4 hours and were harvested and washed with cold PBS containing 0.5% bovine serum albumin

(BSA) and 0.1% sodium azide. Cell preparations were then incubated with a saturating amount (0.5 μg/reaction) of anti-neu mAb 7.16.4 or anti-EGFR mAb 425, or an irrelevant mAb (such as 9BG5 against the hemagglutinin of Reovirus receptor), at 4°C for 30 min and stained with FITC-conjugated anti-mouse IgG (Sigma) for another 30 min. Cells were then fixed with 2% paraformaldehyde and analyzed by flow cytometry (FACScan, Becton-Dickinson), as described previously (43). Briefly, after subtracting the non-specific background staining with 9BG5, the mean channel values from each time point were used to determine the percentage of surface expression of EGFR or $p185^{neu}$ proteins at the various time points after EGF treatment.

Expression of EGFR and/or mutant $p185^{neu}$ proteins

Stably transfected cell lines expressing the EGFR and various $p185^{neu}$ deletion mutant proteins derived from full length transforming $p185^{neu}$ (designated Tneu) were generated in the NR6 cell background (44). Stable transfectants derived from NR6 fibroblasts expressing human EGFR (termed NE91 cells) were generated. NE91 cells, as well as NR6 parental cells, were then transfected with various $p185^{neu}$ cDNA constructs to express one of the following mutant $p185^{neu}$ proteins with or without EGFR, respectively: (a) Er/Tneu or Tneu (full-length oncogenic p185neu product); (b) Er/T691stop or T691stop (lacking 591 aa from the carboxyl-terminal); (c) Er/TAPstop or TAPstop (a 122-aa truncation at the carboxyl terminus), and (d) Er/TΔ5 or TΔ5 (an ectodomain deleted p185neu product).

B104-1-1 murine fibroblasts transformed by the expression of oncogenic $p185^{neu}$ were used as a positive control since surface expression of $p185^{neu}$, biochemical features of p185neu homodimerization, and $p185^{neu}$ transforming potency have been characterized previously (13, 22, 27). As shown in Table 1, relative expression levels of various $p185^{neu}$ mutant proteins in selected clones were estimated by a comparison with B104-1-1 cells, while the expression of EGFR in these cells was estimated by Scatchard analysis. In order to observe an enhancement of EGFR-mediated cellular signalling and transformation, clone Er/Tneu expressing a moderately low level of both receptors (~10^4/cell) was chosen. In other subclones, the expression of EGFR and/or mutant $p185^{neu}$ proteins was approximately 10^5/cell.

Results:

Tyrosine kinase activity in living cells

Previous studies showed that EGF, in an EGFR-dependent manner, stimulated phosphorylation of the $p185^{neu}$ and c-erbB-2 gene products with a concomitant increase in their tyrosine kinase activities (47-50). Heterodimerization of p185 and the EGFR facilitates cross-phosphorylation (24, 25) since a full-length, kinase-deficient $p185^{neu}$ mutant (K757M) is trans-phosphorylated upon association with holo-EGFR (37).

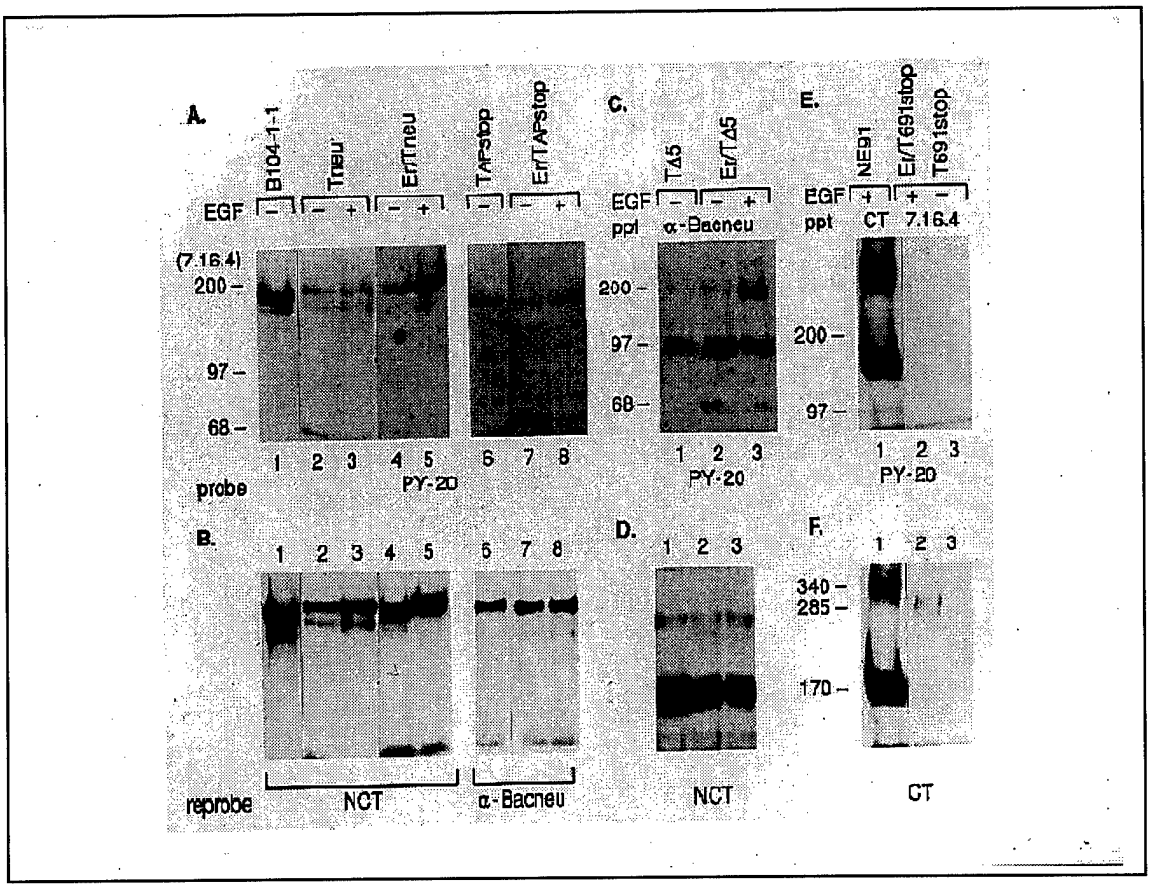

Figure 1. Tyrosine phosphorylation of EGFR and mutant p185neu proteins in living cells.
Cells in panels **A, C,** and **E** were treated with or without EGF as indicated. Cells in panel E were also treated with the chemical cross-linker BS^3 (2mM). Cell lysates were then immunoprecipitated with anti-neu antibodies 7.16.4 or α-Bacneu or with anti-EGFR CT as indicated. Proteins were separated by 6% (A and C) or 4-8% (E) gradient SDS-PAGE followed by immunoblotting with anti-phosphotyrosine mAb PY-20. After stripping the PY20 signals presented in the top panels, these nitrocellulose membranes were reprobed with anti-neu NCT (**B** lane 1-5, and **D**), α-Bacneu (**B** lane 6-8) or α-EGFR CT (**F**) to compare protein amounts used in each sample.

We examined the tyrosine phosphorylation level of $p185^{neu}$ derivatives in living cells in response to EGF treatment. After the addition of EGF, oncogenic Tneu and its derivatives were immunoprecipitated by anti-neu antibodies, and receptor phosphotyrosine content *in vivo* was detected by immunoblotting with an anti-phosphotyrosine antibody (PY20) (Fig. 1). Full-length Tneu from control B104-1-1 fibroblasts displayed constitutive kinase activity (Fig. 1). Upon EGF stimulation, there was indeed a further increase in phospho-tyrosine content of Tneu in Er/Tneu cells expressing lower amounts of the Tneu protein (Fig.1A, lane 4 and 5), but not in cells expressing Tneu alone (lane 2 and 3). A weak tyrosine phosphorylation signal was detected in TAPstop cells (Fig. 1A lane 6). EGF stimulation did not appreciably increase the tyrosine phosphorylation of TAPstop in EGFR co-expressing cells (Fig. 1A, lane 7 and 8), although the association of EGFR and TAPstop was evident.

Truncation of the Tneu carboxyl terminus, and deletion of at least three known critical tyrosine residues, was associated with the failure to trans-phosphorylate the p185neu mutant protein. Elimination of the ectodomain did not impair the intrinsic kinase activity of Tneu-derived TΔ5, since the TΔ5 mutant receptor was still a competent tyrosine kinase (Fig.1C, lane 1). However, unlike the full-length Tneu, no further increase in tyrosine phosphorylation of TΔ5 was detected in Er/TΔ5 cells with EGF stimulation (Fig. 1C, lane 2 and 3). The EGFR in Er/TΔ5 cells was autophosphorylated by EGF treatment, since the anti-Bacneu antisera also immunoprecipitated the EGFR (Fig. 1C, lane 3). These results correlated with our failure to detect physical interactions between EGFR and TΔ5 proteins (Not shown).

We next analyzed tyrosine kinase activation in EGFR-positive NE91 cells with or without T691stop neu co-expression. Treatment with EGF and a chemical cross-linking reagent resulted in heavy tyrosine phosphorylation of EGFR monomers and homodimers in NE91 cells (Fig. 1E, lane 1). No detectable tyrosine phosphorylation of cytoplasmic domain-deleted T691stop neu was seen in cells with or without EGFR co-expression (Fig. 1E, lane 2 and 3, respectively). However, the tyrosine phosphorylation signal of an intermediate band (~285 kDa) representing EGFR-T691stop heterodimeric complex was also undetectable (Fig. 1E, lane 2), although a significant portion of EGFR forms a heterodimer with T691stop under these conditions. Tyrosine kinase activation of full-length EGFR was thus completely inhibited when the EGFR was physically associated with the T691stop neu mutant protein. These results are consistent with the observation from cells co-expressing the EGFR with N691stop neu derived from normal $p185^{neu}$ (37).

These experiments further support our model that the heteroreceptor assembly mediated primarily by ectodomain interactions facilitates kinase trans-activation and trans-phosphorylation occurring as a result of interactions between cytoplasmic domains (27, 37).

EGF-induced receptor down-regulation from the cell surface

Numerous studies suggest that ligand-mediated receptor endocytosis and degradation is a kinase-dependent process for many types of growth factor receptors (55). We found that the efficiency of receptor down-regulation and degradation in cells co-expressing the EGFR and $p185^{neu}$ correlated well with heterodimeric kinase activities (37). We used this method as an alternative assay to examine the kinase activity of various heterodimers.

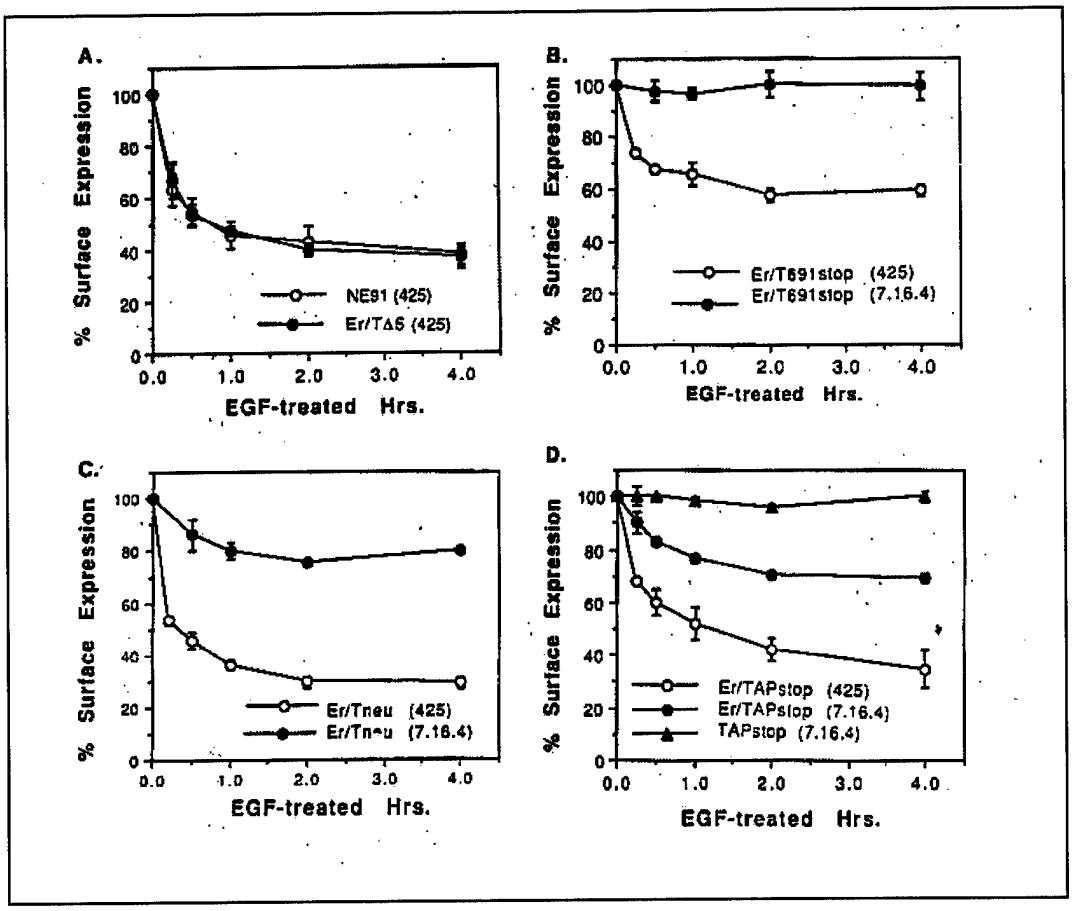

Figure 2. EGF-mediated receptor down-regulation.
Cells were plated in 6-well dishes overnight and treated with EGF (50 ng/ml) for 0-4 hours at 37°C. Cells were then washed with FACS buffer and stained with anti-neu mAb 7.16.4 or anti-EGFR mAb 425 as indicated. After subtracting the background staining with irrelevant mAb 9BG5, the percentage of cell surface receptor expression reflected by the mean fluorescent intensity from each treated sample verses that from a non-treated sample was plotted against EGF treatment time. (**A**) NE91 and Er/TΔ5, (**B**) Er/T691stop, (**C**) Er/Tneu, and (**D**) TAPstop and Er/TAPstop.

Cells were incubated with EGF (50 ng/ml) for various times prior to cell surface staining with anti-neu mAb 7.16.4 or anti-EGFR mAb 425 followed by the staining with FITC-conjugated anti-mouse-IgG. Cell surface expression of each receptor was analyzed using flow cytometric analysis. EGF treatment of NE91 cells (expressing EGFR only) resulted in a reduction of cell surface EGFR after 15 min and disappearance of over 60% of EGF receptors from the cell surface after 4h (Fig.2A). Normal EGFR down-regulation was not affected by the co-expression of kinase-active truncated TΔ5, as the efficiency of EGFR down-regulation in Er/TΔ5 cells was very similar to that seen in NE91 cells (Fig. 2A). A similar EGFR down-regulation curve was observed in Er/Tneu and Er/TAPstop cells (Fig. 5C and 5D, respectively), indicating that the EGFR behaves as an active receptor kinase in these cells. Moreover, about ~20% of Tneu or 25% TAPstop was co-downregulated with the

EGFR upon EGF stimulation (Fig. 2C and 2D). As illustrated above, the low expression of Tneu and the EGFR in Er/Tneu cells was insufficient to demonstrate the physical association of the two receptors biochemically. The current assay was more sensitive in determining EGF-mediated receptor interactions. Control cells expressing TAPstop alone did not respond to EGF treatment and the surface expression of TAPstop remained unchanged within the period of EGF treatment (Fig. 2D).

Analysis using an EGF-mediated pulse-chase assay showed that the down-regulated EGFR and co-down-regulated TAPstop proteins efficiently went into the degradation pathway (data not shown), similar to the cells overexpressing EGFR and $p185^{neu}$ (37). Our data suggested that EGFR and either Tneu or TAPstop associated into an active kinase complex and that these receptor assemblies exhibited comparable kinetics of receptor endocytosis.

Co-expression of T691stop with EGFR, however, resulted in diminished EGF-induced down-regulation of EGFR. The maximal reduction of surface EGF receptor was ~35% of the total at the latest time points examined. In addition, no detectable co-down-regulation of the cytoplasmic domain deleted T691stop was observed in Er/T691stop cells (Fig. 2B), correlating with the observed inactive heterodimer of EGFR/T691stop (Fig. 1E and 1F). This finding supports the idea that receptor down-regulation is coupled to receptor tyrosine kinase activity. The formation of the inactive heterodimer between the EGFR and T691stop neu proteins influenced the overall kinetics of EGFR down-regulation.

Transforming potency of cells expressing endodomain mutant p185neu proteins with or without EGFR

Kinase activity of $p185^{neu}$ is required for its cellular transforming activity (13). The crucial role of tyrosine phosphorylation of the carboxyl terminus of $p185^{neu}$ in cellular transformation has also been illustrated by alteration of several tyrosine residues (56) or large structural deletions, such as seen with TAPstop (42), since these mutations within $p185^{neu}$ greatly reduced oncogenicity. Transforming ability of ectodomain-deleted TΔ5 was less potent than full-length Tneu, possibly due to the reduced efficiency of forming active receptor complexes when compared with full-length oncogenic $p185^{neu}$ (27).

We examined whether co-expression of the EGFR with Tneu and its derivatives could enhance transforming efficiency compared to cells expressing these mutant p185neu proteins alone. Cells were analyzed by focus formation and anchorage-independent growth and representatives are shown in Figure 3. All cell lines (except kinase-deficient T691stop and Er/T691stop clones) were able to form foci independent of ligand stimulation (Fig. 3A). Co-expression of the EGFR with Tneu in Er/Tneu cells increased the ability to form foci, both in density and absolute number. However, co-expression of the EGFR with kinase-active truncated mutant TAPstop or TΔ5 did not enhance focus formation efficiency in Er/TAPstop and Er/TΔ5 cells when compared with TAP/stop and TΔ5 cells, respectively.

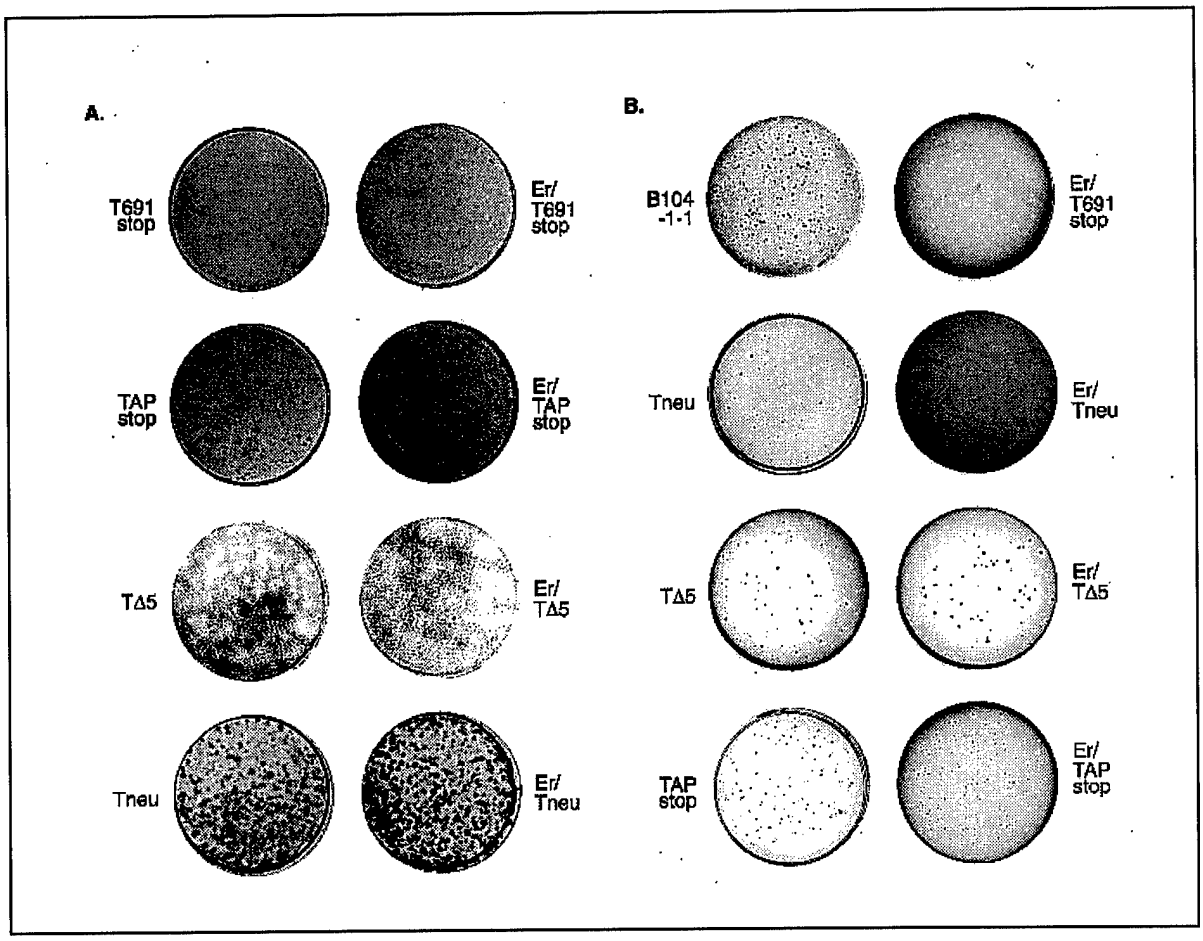

Figure 3. Focus formation and anchorage-independent growth.
A. Ten thousand cells per dish of stably transfected subclones expressing mutant Tneu derivatives (with or without EGFR background) were cultured in DMEM/2% FBS. Cells were fixed with formalin and stained with hematoxylin on day 21. **B.** One thousand cells per dish of B104-1-1 cells and various other mutant Tneu expressed cells were seeded in soft agar as described in Experimental Procedures. Cells were supplemented with DMEM containing 5% FBS once a week. Colonies in the entire dish were photographed after the 3-week culture period. (See appendix)

Colony growth in soft agar (Fig. 3B) employed B104-1-1 cells as a positive control, while the Er/T691stop served as a negative control and did not exhibit transformed foci under the same conditions. Tneu cells expressing lower amounts of oncogenic $p185^{neu}$ than B104-1-1 cells formed colonies, but less efficiently. However, more colonies were observed in EGFR co-expressed Er/Tneu cells. Co-expression of the EGFR with Tneu still permits functional heterodimerization in addition to homodimerization of either receptor, resulting in elevated biological activity, contributing to increased transforming activity in vitro. Cells expressing kinase-active truncated mutant TAPstop or TΔ5 mutant proteins alone displayed reduced colony growth efficiency in soft agar when compared with control B104-1-1 cells, although the expression levels of $p185^{neu}$ variants in these cells were similar.

Critically, co-expression of the EGFR with TΔ5 or TAPstop did not increase colony growth efficiency in soft agar. The failure of distinct endodomain interactions between $p185^{neu}$ and the EGFR because of an ectodomain deletion (TΔ5 mutant), or the lack of a functional C-terminus (TAPstop mutant), clearly impairs signalling needed for transformation. The colony growth efficiency of these clones is summarized in Table 1.

Table 1. Transformation Parameters and Relative Receptor Expression Levels of Cell Lines

Cells	Colonies in soft agar	Tumor growth			Receptor expression	
	(% efficiency)	incidence	latency (wk)	mm3 (at wk 6)	neu protein	EGFR
B104-1-1	33.7 ± 0.6	6/6	1	sacrificed	1.5×10^5	0
NE91	<0.1	0/4	-	NT	0	2.8×10^5
Tneu	5.4 ± 0.4	4/4	4-5	158	3.7×10^4	0
Er/Tneu	10.2 ± 0.6	6/6	2-2.5	663	3.9×10^4	3.8×10^4
T691stop	<0.1	0/4	-	NT	4.5×10^5	0
Er/T691stop	<0.1	0/4	-	NT	4.3×10^5	2.5×10^5
TAPstop	6.5 ± 0.5	6/6	4-5	177	1.2×10^5	0
Er/TAPstop	5.5 ± 0.6	4/4	4-5	168	1.3×10^5	4.6×10^5
TΔ5	5.7 ± 0.3	6/6	3	545	ND	0
Er/TΔ5	6.6 ± 0.4	4/4	3	593	ND	1.6×10^5

The number of EGF receptors on NE91 and other transfected cells was determined by Scatchard assays. Cell surface expression of neu proteins was estimated by comparing the mean channel fluorescent intensity with that from B104-1-1 cells using flow cytometry analysis. $p185^{neu}$ on B104-1-1 cells was originally determined by an ^{125}I-labeled anti-neu mAb binding assay (22). For the tumor growth assay, individual clones (1×10^6 cells per site) were injected intradermally into athymic mice. NT, no tumor after 10 weeks; ND, not determined.

Objective 1 summary:

The results indicate that $p185^{neu}$/EGFR heterodimerization is highly favored, and is preferred even when $p185^{neu}$ homodimerization is facilitated by the neu transmembrane point mutation. Together with the recent observation that erbB-2 is the preferred heterodimerization partner of all erbB members (36), these studies emphasize that neu/erbB-2 may mediate signalling diversity through structural interactions governed by particular ectodomain sequences. For instance, erbB-3 is a less active kinase than other erbB proteins (58), but it serves as a binding site for NDF (28) and forms a potent heterodimer with erbB-2, consequently engaging various downstream substrates. Neu/erbB-2 may not be required for ligand binding, but may reconstitute signalling by laterally engaging other erbB proteins in a preferred manner.

Recent studies showed that the signal adapter Grb2 is required for efficient endocytosis of EGFR (59), and selective and regulated signal transduction from receptor tyrosine kinases (RTK) may continue within the endosome (60). Interestingly, RTK-mediated activation of extracellular-regulated kinases (ERKs) may also involve endocytotic trafficking since inhibition of clathrin-mediated endocytosis has recently been shown to impair rapid EGF-stimulated activation of ERKs (61). Therefore, it is reasonable to speculate that EGF-induced endocytosis of these receptor complexes reflects both heterodimeric kinase activity and the efficiency of activating downstream signalling components.

Co-transfection of kinase-deficient full-length Tneu (K757M) with C-terminal-truncated TAPstop did enhance cell transformation by an intermolecular association and signal amplification mechanism (27). These data emphasize that cooperative signalling requires not only the formation of an active kinase complex, but also functional carboxyl-termini within the two receptor endodomains which recruit various downstream molecules required to generate signals mediating cell growth and transformation.

These results further support the notion that cooperative signalling of the $p185^{neu}$/EGF receptors requires the ectodomain for ligand-mediated physical association, while the endodomain provides contacts for efficient intermolecular kinase activation, and the phosphorylated carboxyl termini is essential for recruiting particular cellular substrates required for signal diversification and down regulation. These features are probably characteristics that are used by many receptor ensembles involved in enzymatic signalling in cells.

Cloning of NAF

We have employed a degenerate RT-PCR method to identify neuregulin-related genes in the ATL-2 cell line and in other tissues. Multiple primers corresponding to several conserved regions in the neuregulin 1 and 2 genes were designed to identify other genes that contain the conserved motifs.

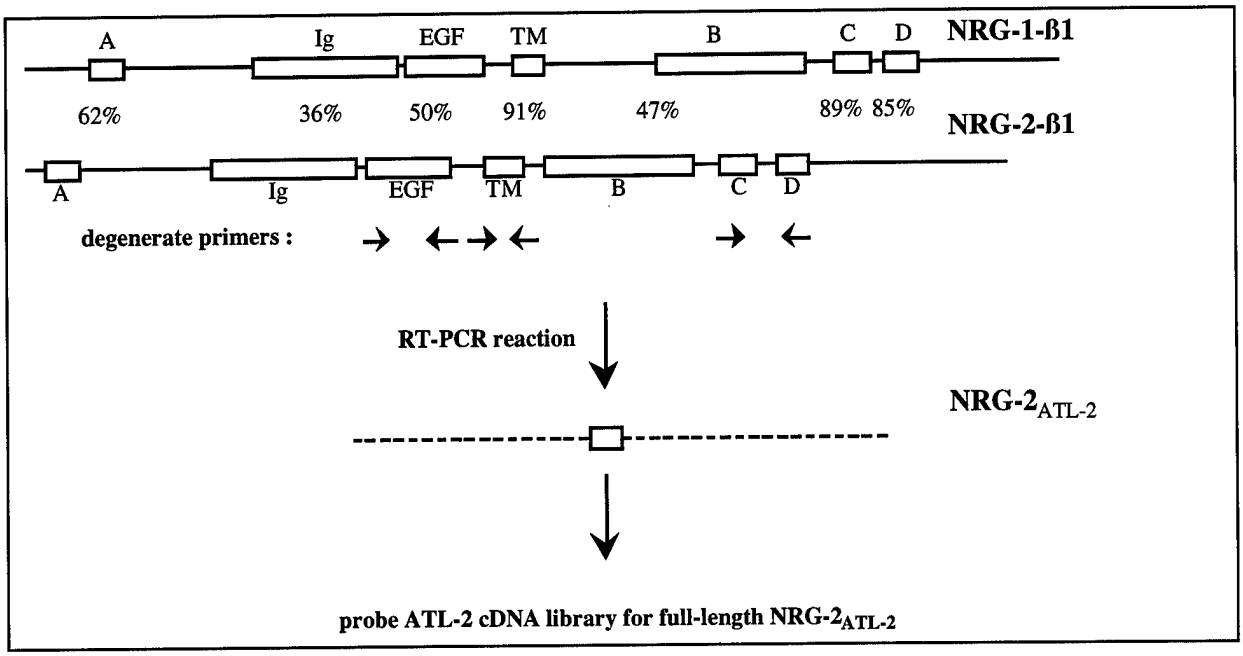

Figure 4. Neuregulin schematic and RT-PCR strategy.
These primers are indicated in the schematic and all three pairs were initially used to survey the transcript sequence space from the adult rat kidney and brain and from a human T cell line in which we have previously described the existence of a neu protein-specific activating factor designated NAF.

```
db1|AB005060|AB005060 Homo sapiens mRNA for NRG-2, complete cds
            Length = 3020

Query:   106 AGGCTGAGGAATTGTACCAGAAGAGGGTCCTGACCATCACGGGCATCTGCGTGGCTCTGC 47
             | || |||||  ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1415 AAGCCGAGGAGCTGTACCAGAAGAGGGTCCTGACCATCACGGGCATCTGCGTGGCTCTGC 1474

Query:    46 TGGTCGTGGGCATCGTCTGTGTGGTGGCCTACTGCAAGACCAAAAA 1
             |||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1475 TGGTCGTGGGCATCGTCTGTGTGGTGGCCTACTGCAAGACCAAAAA 1520


Identities = 102/106 (96%)
```

Figure 5. Alignment of NRG-2ATL-2 with the corresponding NRG-2 sequence.
This product showed the closest homology to the transmembrane region of the NRG-2 gene product (64) at the nucleotide level (97% across 106 nucleotides).

We have identified a neuregulin or NRG-like gene using the NRG transmembrane primers. The level of homology illustrated in Fig. 5 suggests that

we have identified a human NRG-2 or NRG-2-like gene. Expression in the ATL-2 cell line of our RT-PCR fragment was 97% identical at the nucleotide level (102/106 nucleotides) and 100% identical (34/34) at the amino acid level with the reported NRG-2 transmembrane region. The minor differences at the nucleotide level may reflect a sequencing artifact, Taq polymerase infidelity, a naturally occurring polymorphism in the human NRG-2 gene, or a possible ATL-2-derived sequence that is distinct from NRG-2.

The NAF activity we described from the ATL-2 cell line may not correspond to the known NRG-2 proteins that bind only the erbB-3 and erbB-4 RTKs but may represent a previously uncharacterized NRG-2 splice variant. Alternatively, the ATL-2-derived NRG-2 related sequence may represent a novel gene. It is also possible that the NAF activity is not related to the NRG sequences we have identified in the ATL-2 cell line. We have argued that NAF is truly p185 specific because it causes a dramatic increase in $p185^{neu/erbB2}$ homodimerization and surface down-regulation and has no effect on neu extracellular domain deletion mutants (61). It is possible that we may have identified a new member of the NRG gene family.

To evaluate the nature of the NRG-2-related sequences expressed in the ATL-2 cell line and determine whether any of these isoforms corresponds to the NAF activity that we previously described, we are in the process of cloning cDNAs that correspond to the NRG-2-related fragment we have obtained. We are using the NRG-2 (ATL-2) probe to clone the full length NRG-2-related gene from an ATL-2 cDNA library. We have generated an oligo-dT primed cDNA library in phage lambda gt11 from ATL-2 poly-A+ RNA obtained from conditions in which the NAF activity was identified.

Several isolates will be characterized to ascertain whether there is one or several NRG-2-related splice variant(s) or novel species expressed in the ATL-2 cell cDNA library. Several clones have been identified in a primary screen of the ATL-2 cDNA library and these candidates are being plaque purified. Full length clones, once obtained, will be expressed in several systems and isolated for studies of kinase activation and for p185-specificity using in vivo and in vitro kinase assays.

Tasks 1-3 in the Statement of Work have been completed. Task 4, the detailed analysis of potential genes that may encode NAF, has begun.

CONCLUSIONS

The results indicate that $p185^{neu}$/EGFR heterodimerization is highly favored, and is preferred even when $p185^{neu}$ homodimerization is facilitated by the neu transmembrane point mutation. Together with the recent observation that erbB-2 is the preferred heterodimerization partner of all erbB members (36), these studies emphasize that neu/erbB-2 may mediate signalling diversity through structural interactions governed by particular ectodomain sequences.

These data emphasize that cooperative signalling requires not only the formation of an active kinase complex, but also functional carboxyl-termini within the two receptor endodomains which recruit various downstream molecules required to generate signals mediating cell growth and transformation.

These results further support the notion that cooperative signalling of the $p185^{neu}$/EGF receptors requires the ectodomain for ligand-mediated physical association, while the endodomain provides contacts for efficient intermolecular kinase activation, and the phosphorylated carboxyl termini is essential for recruiting particular cellular substrates required for signal diversification and down regulation.

Finally, our new data indicate the likely existence of new forms of Neuregulin that may represent the authentic p185 ligand.

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APPENDIX

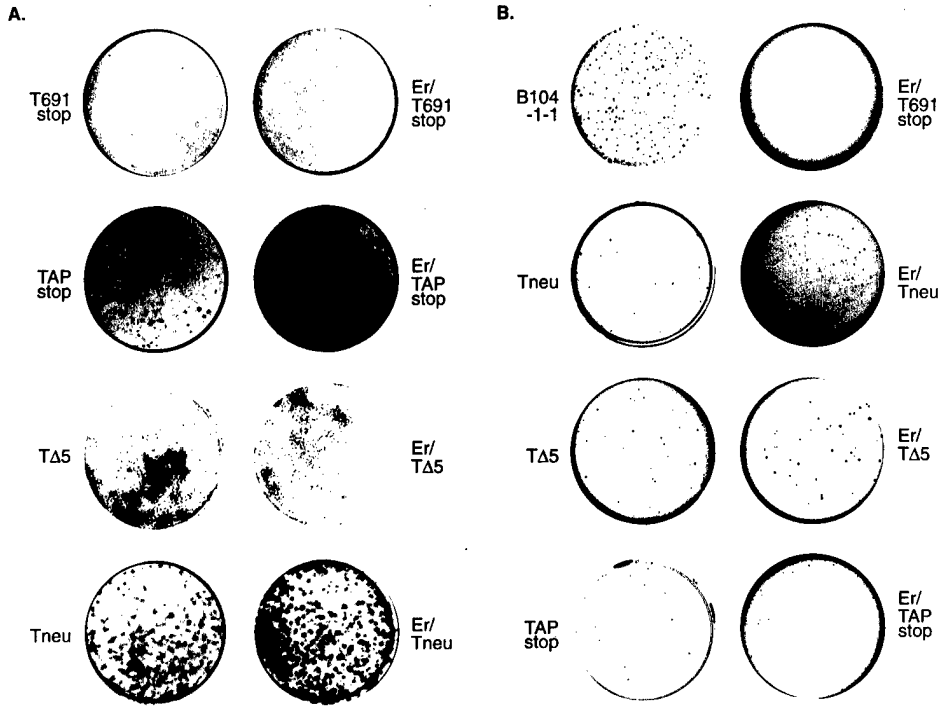

Figure 3. Focus formation and anchorage-independent growth.

A. Ten thousand cells per dish of stably transfected subclones expressing mutant Tneu derivatives (with or without EGFR background) were cultured in DMEM/2% FBS. Cells were fixed with formalin and stained with hematoxylin on day 21. **B.** One thousand cells per dish of B104-1-1 cells and various other mutant Tneu expressed cells were seeded in soft agar as described in Experimental Procedures. Cells were supplemented with DMEM containing 5% FBS once a week. Colonies in the entire dish were photographed after the 3-week culture period.